ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Feb. 1992, p. 502–504
0066-4804/92/020502-03$02.00/0

Vol. 36, No. 2

Conjugal Transfer of Plasmid DNA from *Enterococcus faecalis* to *Escherichia coli* in Digestive Tracts of Gnotobiotic Mice

FLORENCE DOUCET-POPULAIRE,[1]† PATRICK TRIEU-CUOT,[2]‡ ANTOINE ANDREMONT,[1,3] AND PATRICE COURVALIN[2]*

Laboratoire d'Ecologie Microbienne, Institut Gustave-Roussy, 94805 Villejuif Cedex,[1] Unité des Agents Antibactériens, Institut Pasteur, 75724 Paris Cedex 15,[2] and Faculté de Pharmacie, Chatenay-Malabry,[3] France

Received 24 June 1991/Accepted 22 November 1991

We have studied the transfer of the conjugative shuttle plasmid pAT191, which confers resistance to kanamycin, from *Enterococcus faecalis* to *Escherichia coli* in the digestive tracts of six gnotobiotic mice. Colonies of *E. coli* resistant to kanamycin were isolated from the feces of two mice, respectively, on days 25 and 35 after the beginning of the experiment and never thereafter. The transfer frequency of pAT191, expressed as the number of transconjugants per donor cell isolated from intestines of sacrificed mice, was 3×10^{-9}. These results indicate that conjugation is a mechanism that could account for the resistance gene flux from gram-positive to gram-negative bacteria observed in nature.

Over the past two decades, we have witnessed the evolutionary response of bacteria to the selective pressure imposed by the medical and veterinary use of antibiotics. Studies on the emergence and dissemination of drug resistance genes have vividly illustrated the genetic flexibility of bacteria. Dissemination of antibiotic resistance appears to be unlimited since resistance genes from gram-positive cocci (staphylococci, enterococci-streptococci) have been detected in various gram-negative pathogens (8, 9, 15) including members of the family *Enterobacteriaceae* (2, 4). In an attempt to elucidate the mechanism by which genetic information is transmitted horizontally between phylogenetically remote organisms, we have constructed various specialized shuttle vectors and demonstrated that plasmid DNA can be conjugatively transferred from gram-positive to gram-negative bacteria (13) and vice versa (14). The ability of the streptococcal conjugative transposon Tn*916* to cross the hypothesized barrier between gram-negative and gram-positive organisms has been demonstrated recently by using a natural transfer system (3). These findings strengthened our proposal that enterococci-streptococci can serve as a reservoir of resistance genes for gram-negative bacteria (12). However, because of the low frequency of conjugal transfer observed in these experiments (from 10^{-6} to 10^{-9} transconjugants per donor colony), it remained speculative to consider that conjugation was the actual mechanism by which genetic exchange occurs between gram-positive and gram-negative bacteria under natural conditions. To answer this question, we have studied the transfer of the conjugative shuttle plasmid pAT191 from *Enterococcus faecalis* to *Escherichia coli* in an animal model. Since the intestinal ecosystem is the most probable meeting point for these two bacterial species in nature, plasmid transfer was monitored in the digestive tracts of gnotobiotic mice.

Plasmid pAT191 was previously described (13) and is composed as follows. This 32.5-kb chimeric vector contains the origin of replication and the transfer functions of the broad-host-range enterococcal plasmid pAMβ1, the origin of replication of pBR322, and a kanamycin resistance gene (*aphA-3*) known to be expressed in both gram-negative and gram-positive bacteria. This shuttle vector is self-transferable by conjugation on solid medium from *E. faecalis* BM4110 to *E. coli* K802N::Tn*10*, with an average frequency of 5×10^{-9} per donor colony formed after the mating period (13). In these experiments, transfer of pAT191 is mediated by the *tra* functions of pAMβ1.

Transfer in vivo of pAT191 was conducted in one group of six adult germfree C3H mice (Centre de Selection des Animaux de Laboratoire, Orléans, France). Animals were fed ad libitum with a commercial diet sterilized by gamma irradiation (4 Mrad) and supplied with autoclaved drinking water acidified to pH 3 to prevent bacterial growth. After 12 h without water, germfree mice were inoculated intragastrically with 1 ml of a broth culture containing 10^8 CFU of *E. coli* K802N::Tn*10* and, 8 days later, with 10^8 CFU of *E. faecalis* BM4110 harboring pAT191 (1). Since the number of isolates in mouse feces reflects the number of bacteria in the cecum (6), fecal samples were collected directly at the anuses of the mice 5 times a week to monitor the level of cecal bacterial population. Freshly passed pellets were weighed, homogenized, and serially diluted in physiological saline, and 0.1-ml fractions were plated onto selective media to enumerate donors, recipients, and transconjugants resistant to kanamycin. Every 5 days, six donors and six recipients isolated from the feces of each mouse were used to check the transferability of pAT191 by filter mating. The selective medium for donors, recipients, and transconjugants was brain heart infusion (BHI) agar containing kanamycin (500 μg/ml), tetracycline (8 μg/ml), and tetracycline (8 μg/ml) plus kanamycin (50 μg/ml), respectively. The numbers of donors and recipients ranged from 8.5 to 9.5 $\log_{10}$ CFU/g of feces throughout the experiment (40 days). No significant difference between the numerations of the two populations was observed. Two colonies of *E. coli* K802N::Tn*10* resistant to kanamycin were isolated from the feces of two mice on days 25 and 35 of the experiment, which corresponded in each case to a population level of 2.0 $\log_{10}$ CFU/g of feces (Fig. 1). At the end of the experiment, the mice were killed, the intestinal tracts were removed from

* Corresponding author.
† Present address: Laboratoire Central de Bactériologie-Virologie, Groupe Hospitalier Pitié-Salpétrière, 75651 Paris Cedex 13, France.
‡ Present address: Laboratoire des Staphylocoques et des Streptocoques, Institut Pasteur, 75724 Paris Cedex 15, France.

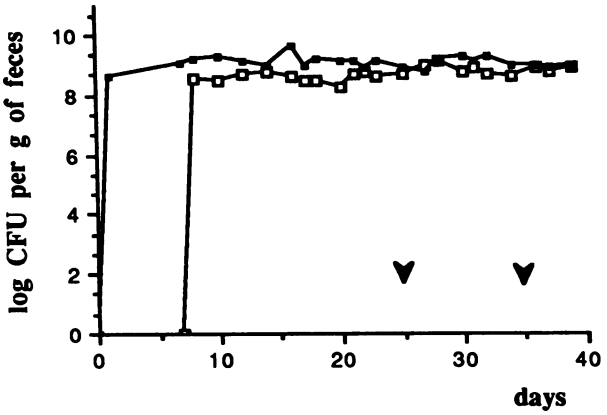

FIG. 1. Bacterial counts in the feces of gnotobiotic mice. Germfree mice were inoculated on day 0 with 10^8 recipient cells (*E. coli* K802N::Tn*10*) and on day 8 with 10^8 donor cells (*E. faecalis* BM4110 harboring pAT191). Symbols: ■, *E. coli* K802N::Tn*10*; □, *E. faecalis* BM4110 harboring pAT191; ▼, *E. coli* K802N::Tn*10* transconjugants. Data are mean $\log_{10}$ counts per gram of feces.

pylorus to rectum, weighed, diluted 10-fold in saline, and homogenized with an ultraturax mixer (Bioblock, Strasbourg, France), and the different populations were enumerated. Total log counts of donor and recipient cells did not differ significantly among individual mice, ranging from 9.8 to 10.5 $\log_{10}$ CFU/g of intestine, and no *E. coli* colonies resistant to kanamycin were isolated. The frequency of in vivo transfer of pAT191 from *E. faecalis* BM4110 to *E. coli* K802N::Tn*10*, expressed as the number of transconjugants per donor cell extracted from intestines of sacrificed mice, was 3×10^{-9}.

The two transconjugants were found to contain 6.4-kb plasmids designated pAT191-Δ1 and pAT191-Δ2. The presence of *aphA-3*, the kanamycin resistance gene carried by pAT191, in these plasmids was confirmed by DNA-DNA hybridization with a specific probe (Fig. 2A). The two plasmids were indistinguishable on the basis of their restriction profiles, and their *Sau*3A-generated fragment patterns were closely related to that of pAT190 (Fig. 2B), the 6-kb gram-negative replicon used to construct pAT191 (13). This indicates that pAT191-Δ1 and pAT191-Δ2 most probably resulted from a deletion(s) which removed most, but not all,

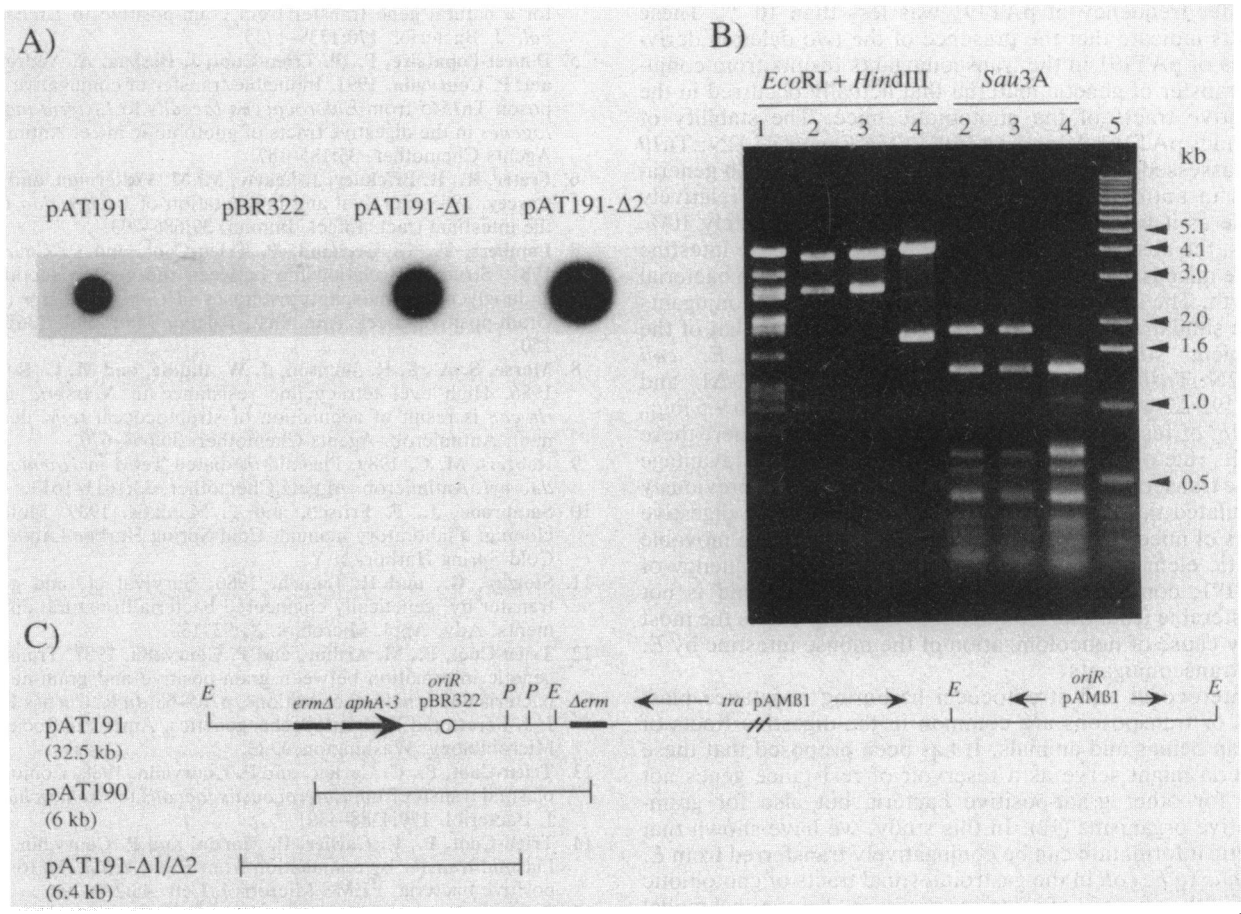

FIG. 2. Structural relationship between plasmids pAT191, pAT191-Δ1, and pAT191-Δ2. (A) DNA of the plasmids indicated was transferred to nytran membranes and probed (10) with a 530-bp *Hpa*II fragment intragenic to *aphA-3* (7) labeled in vitro with ^{32}P (10). DNAs from plasmids pAT191 and pBR322 were used as positive and negative controls, respectively. (B) Agarose gel electrophoresis of DNA of plasmids pAT191 (lane 1), pAT191-Δ1 (lane 2), pAT191-Δ2 (lane 3), and pAT190 (lane 4) is shown. A 1-kb DNA ladder (GIBCO/BRL, Cergy-Pontoise, France) was used as a molecular size standard (lane 5). (C) The structure of the self-transferable shuttle plasmid pAT191 is shown. Portions of pAT191 corresponding to pAT190 and to pAT191-Δ1 and pAT191-Δ2 are indicated by bracketed lines. The components of pAT191 are indicated as follows: *aphA-3*, 3′-aminoglycoside phosphotransferase type III; Δ*erm* and *ermΔ*, 5′- and 3′-truncated *ermB* genes, respectively; *oriR*, origin of replication; *tra*, transfer functions. Abbreviations: *E*, *Eco*RI; *P*, *Pst*I. Only relevant restriction sites are shown (not drawn to scale).

of the pAMβ1 portion of pAT191. Similar molecular rearrangements, although rare, have been observed in pAT191 during the process of in vitro conjugal transfer from *E. faecalis* to *E. coli* (13). A hypothetical location of pAT191-Δ1 and pAT191-Δ2 in pAT191, based on a comparative analysis of their *Eco*RI- and *Pst*I-generated patterns, is shown in Fig. 2C.

E. coli transconjugants resistant to kanamycin were not isolated from the intestines of the six mice at necropsy although they were isolated from the feces of two animals in the course of the experiment. This observation caused us to study the possibility of in vitro transfer of pAT191 onto selective medium after spreading of donors and recipients extracted from the feces. Overnight cultures of donor and recipient were diluted 1:100 into BHI broth devoid of antibiotics. Cells were grown for approximately 5 h, and 200 μl of each culture was spread separately on a 45-μm-pore-size nitrocellulose membrane filter (Millipore Corp.) placed on the top of a BHI agar plate. After 18 h of incubation at 37°C, cells present on each filter were suspended in 1 ml of broth, mixed, and spread on BHI agar containing appropriate antibiotics for selection of donors, recipients, or transconjugants. Under these conditions, no clone of *E. coli* K802N::Tn*10* resistant to kanamycin was isolated and the transfer frequency of pAT191 was less than 10^{-10}. These results indicate that the presence of the two deletion derivatives of pAT191 in the transconjugants results from conjugal transfer of genetic material that actually occurred in the digestive tracts of the gnotobiotic mice. The stability of plasmids pAT191-Δ1 and pAT191-Δ2 in *E. coli* K802N::Tn*10* was assessed by replica plating after growth for 100 generations in antibiotic-free broth. This plasmid was relatively stable as it was lost at a frequency of approximately 10%. Thus, the absence of *E. coli* transconjugants in the intestine of the mice is not due to plasmid segregation during bacterial growth. The in vitro growth rates of the two transconjugants were similar and did not differ significantly from that of the recipient strain (data not shown). Finally, *E. coli* K802N::Tn*10* and *E. coli* harboring pAT191-Δ1 and pAT191-Δ2 were established at similar levels (8.5 to 9.5 $\log_{10}$ CFU/g of feces) in monoxenic mice. Taken together, these results rule out the possibility of an ecological disadvantage of the transconjugant relative to the recipient. We previously postulated that the level of transconjugants in the digestive tracts of mice reflects the transfer frequency of the movable genetic element studied (5). The low transfer frequency of pAT191, combined with the fact that this plasmid is not transferable from transconjugant to the recipient, is the most likely cause of noncolonization of the mouse intestine by *E. coli* transconjugants.

Enterococci and streptococci harboring resistance plasmids or transposons are common in the digestive tracts of human beings and animals. It has been proposed that these bacteria might serve as a reservoir of resistance genes not only for other gram-positive bacteria but also for gram-negative organisms (12). In this study, we have shown that genetic information can be conjugatively transferred from *E. faecalis* to *E. coli* in the gastrointestinal tracts of gnotobiotic mice in the absence of selective pressure. This animal model is probably more favorable to genetic exchange by conjugation than are natural conditions, since it allows intestinal colonization by large numbers of donors and recipients (11). However, it mimics the situation prevailing in the guts of antibiotic-treated human beings and animals, where suppression of the indigenous intestinal microflora allows efficient colonization of the host with nonenteropathic strains of members of the family *Enterobacteriaceae* (11). Conjugation is therefore a mechanism that could account for the resistance gene flux from gram-positive to gram-negative bacteria observed in nature and, in particular, for the presence of the enterococcal-streptococcal erythromycin resistance gene *ermB* in members of the family *Enterobacteriaceae* isolated from patients treated orally with this antibiotic (2).

REFERENCES

1. **Andremont, A., G. Gerbaud, C. Tancrede, and P. Courvalin.** 1985. Plasmid-mediated susceptibility to intestinal microbial antagonisms in *Escherichia coli*. Infect. Immun. **49**:751–755.
2. **Arthur, M., A. Andremont, and P. Courvalin.** 1987. Distribution of erythromycin esterase and rRNA methylase genes in members of the family *Enterobacteriaceae* highly resistant to erythromycin. Antimicrob. Agents Chemother. **31**:404–409.
3. **Bertram, J., M. Stratz, and P. Durre.** 1991. Natural transfer of conjugative transposon Tn*916* between gram-positive and gram-negative bacteria. J. Bacteriol. **173**:443–448.
4. **Brisson-Noel, A., M. Arthur, and P. Courvalin.** 1988. Evidence for a natural gene transfer from gram-positive to *Escherichia coli*. J. Bacteriol. **170**:1739–1745.
5. **Doucet-Populaire, F., P. Trieu-Cuot, I. Dosbaa, A. Andremont, and P. Courvalin.** 1991. Inducible transfer of conjugative transposon Tn*1545* from *Enterococcus faecalis* to *Listeria monocytogenes* in the digestive tracts of gnotobiotic mice. Antimicrob. Agents Chemother. **35**:185–187.
6. **Freter, R., H. Brickner, J. Fekete, M. M. Vickerman, and K. E. Carcey.** 1983. Survival and implantation of *Escherichia coli* in the intestinal tract. Infect. Immun. **39**:686–703.
7. **Lambert, T., G. Gerbaud, P. Trieu-Cuot, and P. Courvalin.** 1985. Structural relationship between the genes encoding 3′-aminoglycoside phosphotransferases in *Campylobacter* and in Gram-positive cocci. Ann. Inst. Pasteur Microbiol. **136B**:135–150.
8. **Morse, S. A., S. R. Johnson, J. W. Biddle, and M. C. Roberts.** 1986. High-level tetracycline resistance in *Neisseria gonorrhoeae* is result of acquisition of streptococcal *tetM* determinant. Antimicrob. Agents Chemother. **30**:664–670.
9. **Roberts, M. C.** 1989. Plasmid-mediated TetM in *Haemophilus ducreyi*. Antimicrob. Agents Chemother. **33**:1611–1613.
10. **Sambrook, J., F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
11. **Stotzky, G., and H. Babich.** 1986. Survival of, and genetic transfer by, genetically engineered bacteria in natural environments. Adv. Appl. Microbiol. **31**:93–138.
12. **Trieu-Cuot, P., M. Arthur, and P. Courvalin.** 1987. Transfer of genetic information between gram-positive and gram-negative bacteria under natural conditions, p. 65–68. *In* R. Curtiss III and J. J. Feretti (ed.), Streptococcal genetics. American Society for Microbiology, Washington, D.C.
13. **Trieu-Cuot, P., C. Carlier, and P. Courvalin.** 1988. Conjugative plasmid transfer from *Enterococcus faecalis* to *Escherichia coli*. J. Bacteriol. **170**:4388–4391.
14. **Trieu-Cuot, P., C. Carlier, P. Martin, and P. Courvalin.** 1987. Plasmid transfer by conjugation from *Escherichia coli* to gram-positive bacteria. FEMS Microbiol. Lett. **48**:289–294.
15. **Trieu-Cuot, P., G. Gerbaud, T. Lambert, and P. Courvalin.** 1985. In vivo transfer of genetic information between Gram-positive and Gram-negative bacteria. EMBO J. **4**:3583–3587.